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Biofuel Cells Select for Microbial Consortia That Self-Mediate Electron Transfer

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Microbial fuel cells hold great promise as a sustainable biotechnological solution to future energy needs. Current efforts to improve the efficiency of such fuel cells are limited by the lack of knowledge about the microbial ecology of these systems. The purposes of this study were (i) to elucidate whether a bacterial community, either suspended or attached to an electrode, can evolve in a microbial fuel cell to bring about higher power output, and (ii) to identify species responsible for the electricity generation. Enrichment by repeated transfer of a bacterial consortium harvested from the anode compartment of a biofuel cell in which glucose was used increased the output from an initial level of 0.6 W m^{-2} of electrode surface to a maximal level of 4.31 W m^{-2} (664 mV, 30.9 mA) when plain graphite electrodes were used. This result was obtained with an average loading rate of 1 g of glucose $liter^{-1}$ day^{-1} and corresponded to 81% efficiency for electron transfer from glucose to electricity. Cyclic voltammetry indicated that the enhanced microbial consortium had either membrane-bound or excreted redox components that were not initially detected in the community. Dominant species of the enhanced culture were identified by denaturing gradient gel electrophoresis and culturing. The community consisted mainly of facultative anaerobic bacteria, such as *Alcaligenes faecalis* and *Enterococcus gallinarum*, which are capable of hydrogen production. *Pseudomonas aeruginosa* and other *Pseudomonas* species were also isolated. For several isolates, electrochemical activity was mainly due to excreted redox mediators, and one of these mediators, pyocyanin produced by *P. aeruginosa*, could be characterized. Overall, the enrichment procedure, irrespective of whether only attached or suspended bacteria were examined, selected for organisms capable of mediating the electron transfer either by direct bacterial transfer or by excretion of redox components.

Biological fuel cells are a potential green energy technology. In a microbial fuel cell bacteria do not directly transfer the electrons which they produce to their characteristic terminal electron acceptor; instead, these electrons are diverted toward an electrode (anode). The electrons are subsequently conducted over a resistance or power user toward a cathode, and thus, bacterial energy is directly converted to electrical energy (35). Three main types of biofuel cells can be distinguished: photoautotrophic-type biofuel cells (38), more common heterotrophic-type biofuel cells, (9) and sediment biofuel cells (3). Biofuel cells have some characteristics that are similar to those of traditional power sources, as well as to those of anaerobic reactors. They can be described on the one hand by electrochemical parameters, such as power density (in watts per square meter of electrode surface), current output, and cell voltage, and on the other hand by biological parameters, such as the nutrient loading rate and biological/chemical oxygen demand (in kilograms per cubic meter per day) (34). However, in contrast to anaerobic reactors, we know relatively little about the microbial ecology of microbial fuel cells (26).

So far, in most of the studies performed the workers used well-defined pure strains, such as *Shewanella putrefaciens* (21, 22) or *Escherichia coli* (13, 30). Recently, two bacteria that exhibited high coulombic efficiency as pure cultures have been described; these bacteria, *Geobacter sulfurreducens* (3, 4) and *Rhodoferax ferrireducens* (7), are capable of transferring the majority of the electrons gained from the carbon sources acetate and glucose, respectively, to the electrode. These studies implied that there was high coulombic efficiency, meaning that there was high electron transfer efficiency. However, this does not imply that there was high energy transfer efficiency, which is dependent on the product of current and potential, both of which determine the energy, expressed in joules. In a limited number of cases, mixed consortia obtained from wastewater treatment plants have been used in flowthrough systems (26). Recently, members of our group described batch systems in which, depending on the organic load, coulombic efficiencies of up to 89% were reached, with 79% energy recovery (34). The crucial parameters for operational effectiveness are (i) bacterial metabolism, (ii) bacterial electron transfer, (iii) performance of the proton exchange membrane, (iv) internal resistance of the electrolytes, and (v) efficiency of the cathode-oxygen electron transfer (20). Most of these parameters have a direct influence on potential losses due to either electron transfer resistance at the electrodes, which are generally described as "overpotentials," or internal resistance of the biofuel cell. Optimization of the fuel cell design can occur through mini-

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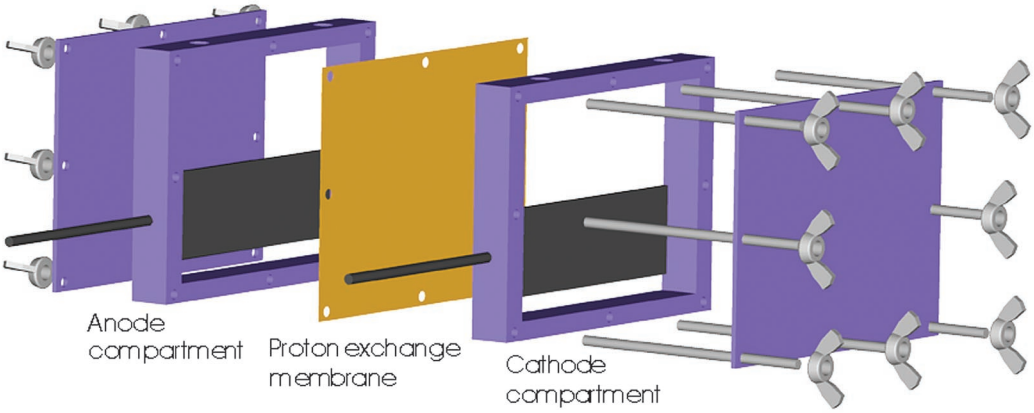

FIG. 1. Schematic diagram of the biofuel cell setup used for the enrichment procedure.

mization of the internal resistance of the cell or reduction of the overpotentials (18). To date, we have only limited knowledge concerning the response of a microbial fuel cell to changes in reactor design.

The most critical step in the biofuel process is the transfer of electrons from the bacteria to the electrode. Bacteria capable of expressing electron chain components in the outer wall are potentially well adapted for use in a microbial fuel cell environment (31), since they provide easy access for electron transfer. In addition, electron mediators need to be added to the medium or to the electrode of biofuel cells in order to obtain higher currents and improved electron transfer (18). Nevertheless, it is known that some bacterial species, such as *Pseudomonas aeruginosa*, can produce compounds like phenazine pyocyanin that function as electron shuttles between the bacterium and an electron acceptor (17).

We postulate that bacteria present in a mediator-less microbial fuel cell self-mediate the electron transfer toward the anode, either with compounds released into the solution or by enhanced membrane-driven electron transfer. The aims of this work were to develop a bacterial consortium with increased energy efficiency and to verify whether membrane-bound or extracellular electron shuttles were correlated with the increase in energy efficiency. The presence of this self-mediating capacity was measured by using cyclic voltammetry, an electrochemical technique optimally suited to determining redox characteristics and hence the electrochemical activity of bacteria. Glucose was always used as the model substrate to enable comparison with results published elsewhere.

MATERIALS AND METHODS

Glucose biofuel cell. A Plexiglas biofuel cell (Fig. 1) was constructed as previously described (34). The volume of the anode compartment and the volume of the cathode compartment were each 240 ml. The anode compartment was flushed with nitrogen gas prior to the experiment. Between the compartments, an Ultrex proton-exchange membrane (Membranes International Inc., Glen Rock, N.J.; 80 cm^2) was installed. The membrane was incubated in 2% NaCl at 45°C for 18 h prior to use. Graphite rods (diameter, 5 mm; Morgan, Grimbergen, Belgium) were used to mount the plate-shaped electrodes. On average, the feeding rate was 1 g of glucose (Sigma, Bornem, Belgium) $liter^{-1}$ day^{-1}.

In the cathode compartment, a graphite electrode (Morgan) was installed. The cathodic electrolyte, prepared as described by Park and Zeikus (32), was a 50 mM $K_3Fe(CN)_6$ solution in 100 mM KH_2PO_4 (adjusted to pH 7 with 1 N NaOH) buffer (Merck, Darmstadt, Germany). For the pure cultures and additional experiments, 45-ml setups were used with the same parameters. Anaerobic, granular, methanogenic sludge was obtained from a potato-processing company (Primeur, Waregem, Belgium). This sludge was mixed and subsequently diluted in nutrient broth (Oxoid, Basingstoke, United Kingdom) to a concentration of 2 g of volatile suspended solids $liter^{-1}$, and 200 ml of this solution was used as the reactor inoculum. After 2 weeks, the inoculum was removed from the reactor, and the bacteria attached to the electrode were scraped off and resuspended in 250 ml of nutrient broth. Two hundred milliliters of this mixture was incubated in the biofuel cell for 7 days, after which the biofilm on the graphite anode was again retained and the suspended bacteria were removed. Such transfers, with omission of the suspended bacteria, were repeated seven times in order to obtain more biomass. After the seventh transfer (day 63), both suspended and attached bacteria were retained. From this point on the reactor medium was refreshed by removing the liquor of the anode compartment from the reactor, centrifuging the suspension (2,500 × *g* for 15 min), and resuspending the bacterial cells in 250 ml of a nutrient broth solution (50 ml was retained for sampling), which served as a general growth medium and the initial electron donor.

To determine the influence of the attached bacteria, after 2 weeks of operation the anode suspension was separated and inserted into an identical biofuel cell with no attached bacteria. Hence, two biofuel cells were obtained; the first cell contained a graphite electrode and suspended bacteria in the anode solution, and the second cell contained the graphite electrode with attached bacteria from the preceding period and new clear medium, as performed previously. The voltage and current were measured simultaneously (see below) in order to calculate the immediate power output.

After this test, the microbial fuel cells were operated for a total of 155 days, starting from the initial inoculation. This created the opportunity to examine the changes that occurred in the microbial community for a longer term. This test was completed with an additional 7-day test with the mixed culture from day 155 (in quadruplicate), during which the power output, the electrochemical activity, and some metabolic parameters were monitored and compared to the parameters for anaerobic control tests in serum flasks.

Metabolic changes. To investigate the influence of the biofuel cell on bacterial metabolism, 100-ml portions of the biofuel cell contents were harvested and placed in serum flasks that were flushed with nitrogen gas. The flasks were operated for 1 week at a loading rate of 1 g of glucose $liter^{-1}$ day^{-1}, during which bacterial growth and metabolism were monitored by gas analysis, plate counting, and volatile fatty acid analysis (see below).

Electrochemical monitoring. Measurements of the power output were obtained by using an Agilent HP 34970 data acquisition unit. Every 30 s, a full-channel scan was performed, and the data were stored. The maximum external system resistance (R) was 100 Ω, and the current (I) (in amps) was deduced as follows: $I = V \times R^{-1} = Q \times t^{-1}$, where V is the voltage (in volts), Q is the charge (in coulombs), and t is the time (in seconds). The power output of the cells (P) (in watts) was calculated as follows: $P = I \times V$. Energy production (E) (in joules) was then expressed as follows: $E = P \times t$.

Chemical analysis. Samples were filtered through a 0.22-μm-pore-size syringe filter unit (Millex; Millipore, Brussels, Belgium). For analysis of the volatile fatty acids, extraction in diethyl ether was performed (14). The samples were analyzed with a capillary flame ionization detector and a gas chromatograph (GC 8000; Carlo Erba Instruments, Wigan, United Kingdom) connected to a computer. The

column used was an Alltech (Deerfield, Ill.) EC-1000 column (length, 30 m; inside diameter, 0.32 mm; d_f, 0.25 μm). The temperature was kept at 135°C for the isotherm oven and at 200°C for the detector and the injector. Nitrogen was used as the carrier gas at a rate of 3 ml min^{-1}. Samples were diluted 10-fold in deionized water, and glucose was assessed by ion chromatography (Dionex Wommelgem, Belgium; Carbopac1 column with borate and amino trap; ED40 pulsed amperometric detection) (15). Gas chromatography (Intersmat IGC 120 MB) was used for determination of the CO_2 and CH_4 contents in the headspace (38). H_2 was measured by using a Microtox exhaled hydrogen monitor (GMI, Wendelstein, Germany), and the detection limit was 5 parts per million by volume (14). For the gas samples, 5 ml of the anode headspace was obtained, after which the biofuel cell was flushed with nitrogen gas. pH electrodes (Metrohm, Herisau, Switzerland) were installed to monitor the compartment pH.

Microscopy analysis. Light microscopy was performed by using a Polyvar microscope (Leitz, Wetzlar, Germany) with a magnification of ×1,000 to verify bacterial growth on electrode surfaces and bacterial morphology. Images were taken digitally by using an Orca IIIm camera (Hamamatsu, Louvain-La-Neuve, Belgium) connected to a personal computer. Digital image analysis was performed by using MicroImage 4.0 and Microsoft ExcelXP. Viability staining (BacLight; Molecular Probes Inc.) was performed to investigate the culture in suspension and on the biofilm. The microscope used was a Zeiss Orthoplan epifluorescence microscope equipped with a mercury short-arc lamp (HBO 100W) with I3 (BP 450/490, LP 515) and N (BP 530/560, LP 580) fluorescence filters for live and dead coloration, respectively, and a ×63 immersion oil objective (N.A. 1.3); very-low-fluorescence immersion oil (Nikon, Badhoevedorp, The Netherlands) was used.

Community analysis. Total DNA was extracted from the sludge samples (6), and 16S rRNA gene fragments were amplified with primers PRBA338fGC and P518r (29) and analyzed by denaturing gradient gel electrophoresis (DGGE) (5). 16S rRNA gene fragments were cut out of the DGGE gel with a clean scalpel and added to 50 μl of PCR water. After 12 h of incubation at 4°C, 1 μl of the PCR water was reamplified with primers P338F and P518r. Five microliters of the PCR product was loaded on a DGGE gel (see above), and if the DGGE pattern showed only one band, it was sent out for sequencing. DNA sequencing of the ca. 180-bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany). The analysis of DNA sequences and homology searches were completed by using standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information along with the BLAST algorithm (1).

Isolation procedure. Bacteria were isolated from the mixed consortium by plating a serial dilution of the consortium from day 155 on nutrient agar. The plates were incubated under aerobic and anaerobic conditions for 2 and 5 days, respectively, and single colonies were purified by using at least two isolation steps until they were morphologically pure, as verified by light microscopy. *Pseudomonas* species were separately isolated and were identified by using King A agar as described by King et al. (23). The pyocyanin produced by *P. aeruginosa* KRP1 was detected spectrophotometrically at 405 nm after extraction with chloroform (11) and by cyclic voltammetry (see below). The 16S rRNA genes of isolates were subsequently analyzed by DGGE as described above and compared with the DGGE pattern of the biofuel cell consortium. For confirmation, the 16S rRNA gene fragments of the pure isolates were amplified by using primers P63F and R1378r (5), sequenced, and analyzed as described above. Strains KRP1, KRA1, and KRA3 are readily available from the LabMET culture collection.

Biofuel cells with pure cultures. Pure cultures of isolates KRP1, KRA1, and KRA3 were prepared in nutrient broth after isolation from the agar plates. The bacteria were counted by plating on nutrient agar. Five milliliters of a fully grown culture was subsequently added to 35 ml of nutrient broth and then placed in serum flasks and in biofuel cells in duplicate to investigate the influence of the electrode on bacterial metabolism. Both reactor types were fed similarly. Biofuel cell power output, gas phase composition, volatile fatty acid production, and electrochemical activity of the bacteria as determined by cyclic voltammetry (see below) were analyzed in 6-day tests. These tests were completed with an additional 7-day test with *P. aeruginosa* isolate KRP1 in quadruplicate to further investigate the voltammetry for this species.

Cyclic voltammetry. Cyclic voltammetry was generally performed by starting from −450 mV and going up to 900 mV and back. If components were oxidized or reduced during this potential sweep of the culture, current peaks appeared on the voltammogram. Every component that could be reversibly oxidized or reduced had a peak on both the upper and lower curves. If one of the peaks disappeared, the component could be regarded as permanently oxidized or reduced. When this technique was used with a bacterial suspension, peaks could appear for both cellular components, such as cytochromes in the periplasm, and excreted redox mediators, such as pyocyanin. The position of the intersection with the x axis of the line between the upper and lower peaks indicated the formal potential of a component, and the size indicated the quantity (2). Prior to the measurements, voltammograms of the mixed consortium were analyzed according to the scan rate and the potential interval used. A scan at a rate of 25 mV s^{-1} between −450 and 900 mV generated clear, reproducible voltammograms with 1.7% variability for surface area expressed as energy (in microjoules). Furthermore, voltammograms were always generated in duplicate for each sample.

For the analysis, 15-ml samples were taken from microbial fuel cells prior to the daily feeding, placed in test vials, and flushed with nitrogen gas prior to measurement. Cyclic voltammetry was performed as described by Park et al. (33). For this a potentiostat (model 263a; Princeton Applied Research, Zele, Belgium) connected to a personal computer (SoftCorr III; Princeton Applied Research) was used at a scan rate of 25 mV s^{-1} in the potential range from −450 to 900 mV. The working electrode was a 5-cm^2 graphite rod that was cleaned in ethanol and deionized water prior to use, the counterelectrode was a platinum wire, and an Ag/AgCl electrode (MF-2052; BAS, Warwickshire, United Kingdom) was used as a reference. All three electrodes were inserted into the test vial, avoiding any contact between the electrodes. Cyclic voltammograms were used to calculate the quantity of redox-active species and the electrochemical activity (E) (in microjoules) by determining the peak size for the voltammogram (P) (in microwatts) (see Fig. 3) and standardizing it to energy as follows: $E = P \times t$, where t is time, which was calculated from $t = \delta V/v$ (δV is the potential difference between the beginning and the end of the peak [in millivolts] and v is the scan rate [25 mV s^{-1}]).

Cyclic voltammetry was performed for the mixed consortium and for four strains isolated from this consortium (isolates KRP1, KRP2, KRA1, and KRA3) in duplicate. The bacteria were grown in nutrient broth in a glucose-fed biofuel cell and tested electrochemically in the spent broth. To obtain a measurement without components released into the solution, the bacteria were centrifuged (10 min at 11,000 × g), resuspended in an equal amount of physiological solution (8.5 g/liter of NaCl solution), and flushed with nitrogen gas for 20 min. Both the resuspended bacteria and the original supernatant were tested by using cyclic voltammetry.

To determine the influence of feeding conditions on the measurements, the consortium was fed glucose (1 g $liter^{-1}$) and subsequently analyzed every 30 min in a 5-h test. After this first test, all other tests were performed with bacteria in the stationary phase. For the mixed consortium, cyclic voltammetry was performed for the consortium in spent broth, for the supernatant, for sterile nutrient broth, and for centrifuged bacteria to determine the effects of the different components.

Nucleotide sequence accession numbers. Nucleotide sequences for bands 2 through 12 and the identified isolates have been deposited in the GenBank database under accession numbers AY483162 to AY483176 and AY489118 to AY489119.

RESULTS

Enrichment of electrochemically active consortia. During the 71-day enrichment period, the power output of the biofuel cells gradually increased. While the recovery of electrons from the carbon source initially was only 4% (maximum power density, 0.65 W m^{-2} of anode surface), electron recovery values of up to 81% were obtained in the subsequent period. Hydrogen concentrations, which initially were up to 43% ± 5% in the headspace, decreased to below the detection limit, while the electron transfer rate increased from 0.65 W m^{-2} to a maximum of 4.31 W m^{-2}. Methanogenesis, which was the key microbial process of the inoculum sludge, was largely suppressed (0.4% ± 0.9% in the headspace) from the beginning of the experiments on. The glucose was completely consumed after 24 h, as verified by ion chromatography. The degradation of the glucose did not result in accumulation of volatile fatty acids in an active microbial fuel cell, indicating that there was further degradation of the volatile fatty acids.

There were no significant differences in power output between the biofuel cells with only suspended bacteria (maximum

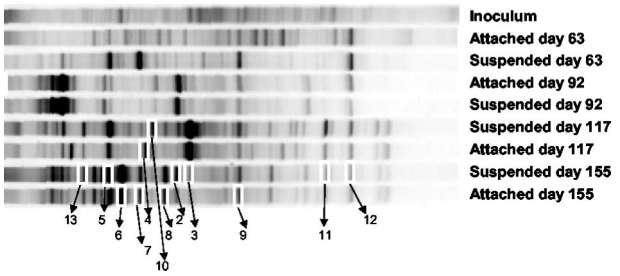

FIG. 2. DGGE pattern of the bacterial consortium as it evolved during the test period, indicating the positions and dominance of several identified species. The band numbers correspond to the band numbers in Table 1.

output, 4.31 W m^{-2} of electrode surface; 664 mV and 30.9 mA) and biofuel cells with only a biofilm on the graphite electrode (maximum output, 3.63 W m^{-2} of electrode surface; 602 mV and 30.1 mA). Live/dead staining revealed that the two setups had comparable amounts of attached and suspended bacteria after 2 days of operation (namely, $8.7 \times 10^{10} \pm 2.7 \times 10^{10}$ live bacteria for the reactor with only suspended bacteria initially and $4.4 \times 10^{10} \pm 1.8 \times 10^{10}$ live bacteria for the reactor with only biofilm initially).

Changes in electron acceptor. Bacteria in a biofuel cell produced less hydrogen than bacteria in a serum flask. In the presence of an electrode the community produced no detectable hydrogen (0.05% ± 0.09% of the headspace at day 155). When the day 155 culture was grown anaerobically in glucose-fed serum flasks containing nutrient broth without an electrode, the hydrogen content of the headspace increased rapidly to 12% ± 3%.

Community analysis. DGGE analysis was performed to monitor the successive enrichment of the microbial culture obtained during the complete experimental period. The band patterns showed that the microbial consortium of the inoculum sludge evolved constantly during the enrichment procedure (Fig. 2). At the end of the enrichment procedure (attached and suspended bacteria on day 92), the banding patterns of the suspended and attached consortia were similar, and the similarity further increased to 95% on day 155. This increase correlated with an increasing power output of the microbial fuel cells. To identify the bacterial species related to good biofuel cell performance, the most dominant bands of the DGGE gel were excised and sequenced at days 117 and 155 (Table 1).

Sequencing results indicated that there was great phylogenetic diversity in the strains identified. Bacteria belonging to the taxa *Firmicutes*, γ-proteobacteria, β-proteobacteria, and α-proteobacteria were present in the microbial fuel cells (Table 1).

Isolation of bacterial strains from the anode consortium. The microbial consortium obtained from a microbial fuel cell after 155 days was plated onto nutrient agar and incubated under both anaerobic and aerobic conditions. A total of 14 species that were morphologically different for anaerobic and aerobic growth were obtained. PCR-DGGE analysis of the isolates was performed. The DGGE bands of isolates P1 and P3, isolates KRA1 and KRAN2, and isolates KRA3, KRAN1, and KRAN3 migrated at the same height as bands 4, 6, and 5, respectively. Sequencing of the 16S rRNA genes of the isolates

TABLE 1. Overview of the bacterial species identified based on either the occurrence of a dominant band in the DGGE pattern or on plating

Band or isolate	Accession no.	Highest homology (accession no.)	% Similarity	No. of identical base pairs	Taxon
Bands					
2	AY483162	*Eubacterium aggregans* (AF073898)	96	114/118[a]	*Firmicutes*
3	AY483163	*Lactococcus lactis* subsp. lactis (AF515226)	100	160/160	*Firmicutes*
4	AY483164	*Pseudomonas* sp. strain ARDRA PSI (AY364085)	99	129/130	*Gammaproteobacteria*
5	AY483165	*Enterococcus gallinarum* CECT9707T (AJ420805)	100	153/153	*Firmicutes*
6	AY483166	*Alcaligenes* sp. strain 2–6 (AY296717)	100	158/158	*Betaproteobacteria*
7	AY483167	Uncultured bacterium clone up. 2 (AY212541)	95	97/102	*Bacteria*
8	AY483168	Swine manure pit bacterium PPC89 (AF445290)	98	69/70	*Bacteria*
9	AY483169	Uncultured earthworm intestine bacterium (AY154530)	97	144/147	*Bacteria*
11	AY483170	*Lactobacillus casei* YDT21 (AF375931)	94	145/153	*Firmicutes*
12	AY483171	*Clostridium* sp. strain MDA2315 (AY238334)	100	127/127	*Firmicutes*
13	AY483172	Uncultured *Enterococcus* sp. clone T8-20 (AF526922)	100	158/158	*Firmicutes*
Isolates					
KRP1	AY483173	*Pseudomonas aeruginosa* ATCC 27853 (AY268175)	95	189/197	*Gammaproteobacteria*
KRP3	AY483173	*Pseudomonas aeruginosa* ATCC 27853 (AY268175)	95	189/197	*Gammaproteobacteria*
KRP4	AY483174	*Bacillus* sp. strain A24 (AF397399)	100	693/693	*Firmicutes*
KRA1	AY483175	*Alcaligenes faecalis* (AF155147)	98	696/704	*Betaproteobacteria*
KRA3	AY489118	*Enterococcus* sp. strain CDC PNS-E2 (AY321376)	99	1,101/1,102	*Firmicutes*
KRA4	AY489119	*Bacillus cereus* ATCC14579 (AF290547)	100	1,101/1,101	*Firmicutes*
KRA5	AY489119	*Bacillus cereus* ATCC14579 (AF290547)	100	1,059/1,059	*Firmicutes*
KRAN1	AY489118	*Enterococcus* sp. strain CDC PNS-E2 (AY321376)	99	1,101/1,102	*Firmicutes*
KRAN2	AY483175	*Alcaligenes faecalis* (AF155147)	98	696/704	*Betaproteobacteria*
KRAN3	AY489118	*Enterococcus* sp. strain CDC PNS-E2 (AY321376)	99	1,101/1,102	*Firmicutes*
KRISO1	AY483176	*Ochrobactrum* sp. strain LMG 20570 (AY040351)	98	664/673	*Alphaproteobacteria*

[a] Number of identical base pairs/number of base pairs examined.

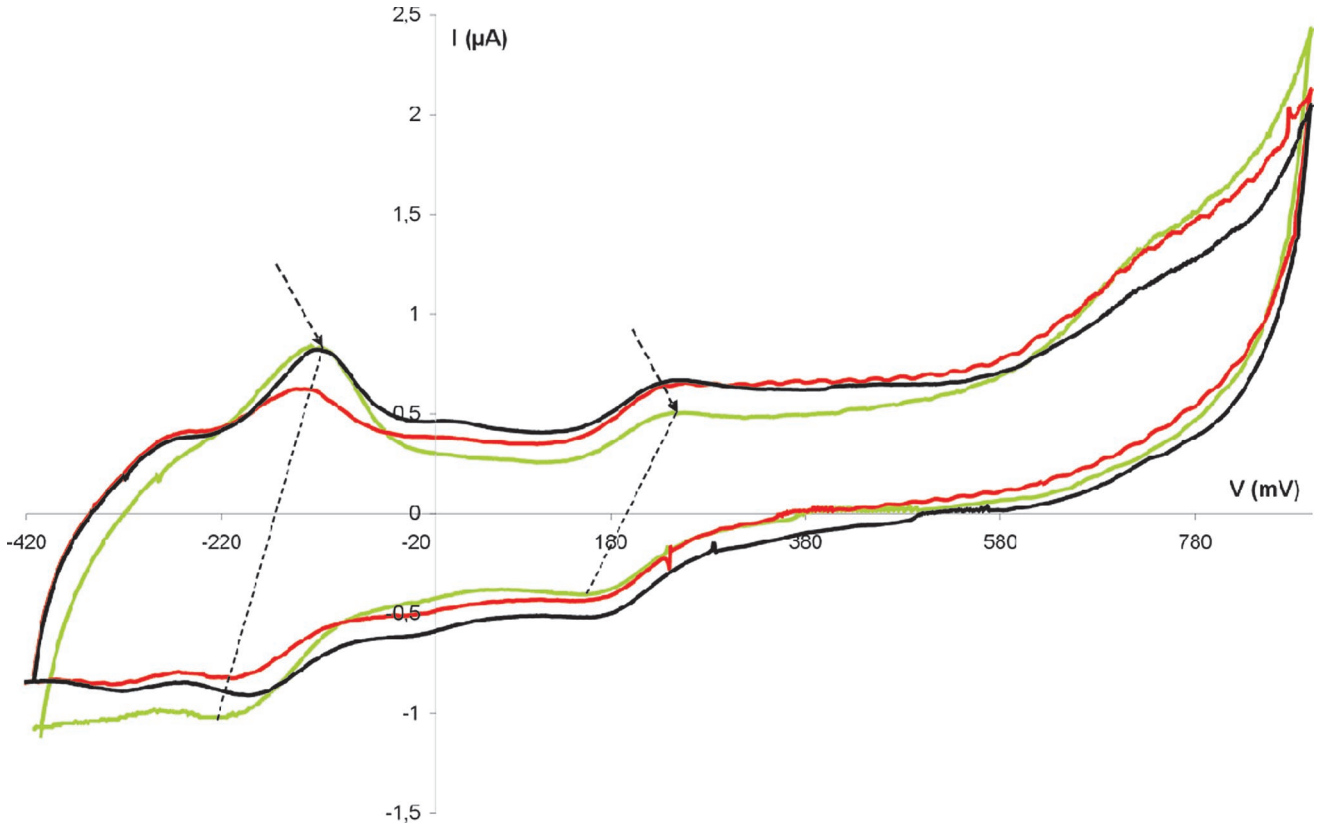

FIG. 3. Cyclic voltammogram of the bacterial consortium after batch feeding with 1 g of glucose liter^{-1}. Black line, voltammogram at time zero; red line, voltammogram after 30 min; green line, voltammogram after 120 min. The dashed arrows and dashed lines indicate the peaks of interest and the intersection with the *x* axis used to determine the component's formal potential. The oxidation-reduction peak at about −180 mV at time zero disappeared after 30 min and then reappeared upon further incubation.

confirmed that the isolates corresponded to the respective bands in the DGGE pattern for the community; thus, a total of six different species were found (Table 1).

Specific plating onto *Pseudomonas* agar (23) confirmed that isolates KRP1 and KRP3 were *P. aeruginosa* isolates. Microscopic evaluation of the consortium confirmed the ubiquitous presence of this rod-shaped bacterium (length, 0.95 μm). Further evidence that this species was present was the blue color of the mixed consortium and of the pure culture when it was grown in a serum flask in *Pseudomonas* broth, when oxygen was present. Extraction of the colorant and spectrophotometric analysis (11) confirmed that the component was blue phenazine pyocyanin, as determined by the absorbance peak in the wavelength scan. When cyclic voltammetry was performed with the purified pyocyanin, a peak with a formal potential of −32 ± 10 mV and with an intensity of 0.31 ± 0.11 μJ was detected for samples obtained from the mixed culture.

Electrochemical activity of the bacteria. When repeated voltammetry was performed with the same sample, the variance of the voltammogram energy content was determined to be 2.0% ± 0.28%. The variance between samples was low for the relative peak size compared to the total voltammogram size, but different bacterial concentrations yielded different voltammogram sizes. Due to this fact, peak sizes for voltammograms for different samples could be regarded only from a qualitative point of view, unless all experimental conditions were identical. However, the variance in the peak position was in the same range as the tolerance of the reference electrode, which was 10 mV. For example, the variance for the peak position for *Enterococcus* was 10.11 mV.

The influence of the bacterial growth phase (lag phase, exponential phase, and stationary phase) on the voltammetric measurements was assessed by obtaining a cyclic voltammogram of the bacterial consortium on day 155 after 1 g of glucose liter^{-1} was added (Fig. 3). At time zero, the voltammogram had oxidation-reduction peaks indicating electrochemical activity. About 30 min after addition of the carbon source, the peak at approximately −170 ± 10 mV had disappeared. This indicated that a redox component active at about −170 ± 10 mV disappeared, but this component reappeared at the full value after 2 h. This shows that there was temporal disappearance of a component out of solution or out of reach of the electrode. The component active at 180 ± 10 mV was apparently not affected by the feeding regimen. The general voltammogram increased with time due to an increase in bacterial density, since bacterial density changes the amount of metabolites in solution, which in turn influences conductivity and capacity. Because of this disturbance of the electrochemical characteristics of the consortium shortly after feeding, additional voltammograms were always obtained for bacteria that were in the stationary phase or were suspended in physiological solution.

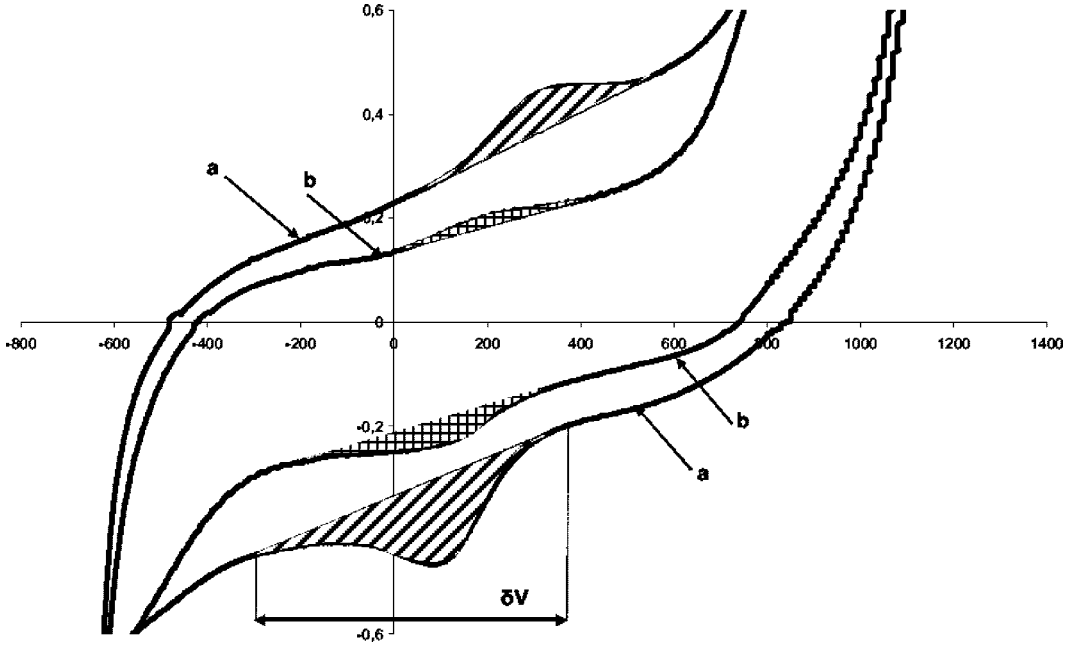

FIG. 4. Different peak sizes, indicated by cross-hatched areas, for cyclic voltammograms of the mixed consortium after 10 days of incubation in a biofuel cell (lines a) and in an anaerobic serum flask (lines b).

Not all peaks consistently appeared on the voltammograms during different test runs. While the peak at 180 ± 10 mV always appeared when the biofuel cell power output was high, the peak at −170 ± 10 mV was not always found, as shown in the voltammograms of the mixed culture in Fig. 4. Biofuel cells without the component that was active at −170 ± 10 mV had a lower power output than the biofuel cells with the component present; i.e., the maximum value was 540 mV at 100 Ω, compared to 664 mV at 20 Ω.

Characterization of the electrochemical activity. Cyclic voltammetry measures both redox activities of components in solution and redox activities of components bound to the bacterial membrane. To determine whether the bacteria were electrochemically active through the compounds released into solution or through contact of the cell wall with the anode, voltammetry was performed with consortia in spent broth and with centrifuged consortia freshly resuspended in physiological solution. No electrochemical activity was observed when fresh nutrient broth and the physiological solution were tested, indicating that the suspension medium used had a limited effect on the measurement. There were two distinct peaks. The first peak, at −170 ± 10 mV, disappeared when the bacteria were centrifuged and resuspended in the physiological solution. To a lesser extent this peak at −170 ± 10 mV was found in the supernatant. The second peak, at 180 ± 10 mV, was always found in the voltammograms of the suspended bacteria in physiological solution. The peak intensity of the voltammograms and thus the electrochemical activity were notably greater when the bacterial consortia were grown in microbial fuel cells than when they were grown in a serum flask (with the same inoculum) (Fig. 4).

Relationship between power output and electrochemical activity. A twofold increase in the power output of a microbial fuel cell correlated directly to about a threefold increase in electrochemical activity of the bacteria a determined by cyclic voltammetry (Fig. 5). To further determine the factors governing the current output, the bacterial consortium was grown in a biofuel cell with glucose for 10 days. The bacterial density, the electrochemical activity calculated from the voltammograms, the power output, and the bacterial metabolism were monitored simultaneously (Fig. 5). After approximately 70 h, an increase in power output was noted, and there was a concomitant increase in electrochemical activity expressed as peak energy (in microjoules), which reached a maximum at approximately 150 h. After this, the power density decreased significantly, and increasing butyric acid concentrations (up to 2.01 g liter^{-1}) were observed. The bacterial concentration in the biofuel cell was greater than the concentration in the anaerobic batch reactor ($1.62 \times 10^9 \pm 1.29 \times 10^8$ and $4.75 \times 10^7 \pm 1.34 \times 10^6$ CFU ml^{-1}, respectively).

Verification of the importance of soluble redox mediators. Pure bacterial cultures can differ in the way that electrons are transferred to the electrode; either soluble components or membrane-bound components are used (Fig. 6). A number of biofuel cell-bred cultures were tested both in spent broth and resuspended in physiological solution after centrifugation. The voltammogram for *P. aeruginosa* had a peak at approximately −100 mV that disappeared when the culture was centrifuged and resuspended in physiological solution. Isolate KRA3 produced a peak at about 200 mV that disappeared after centrifugation and resuspension in physiological solution. The two other species examined in the same way had lower peak intensities. The voltammograms of the centrifuged and noncentrifuged cultures of both species did not differ significantly, indicating that there was limited involvement of dissolved components.

Pure cultures of *P. aeruginosa*, isolate KRA3, and isolate KRA1 were tested for activity in a biofuel cell during 7 days of

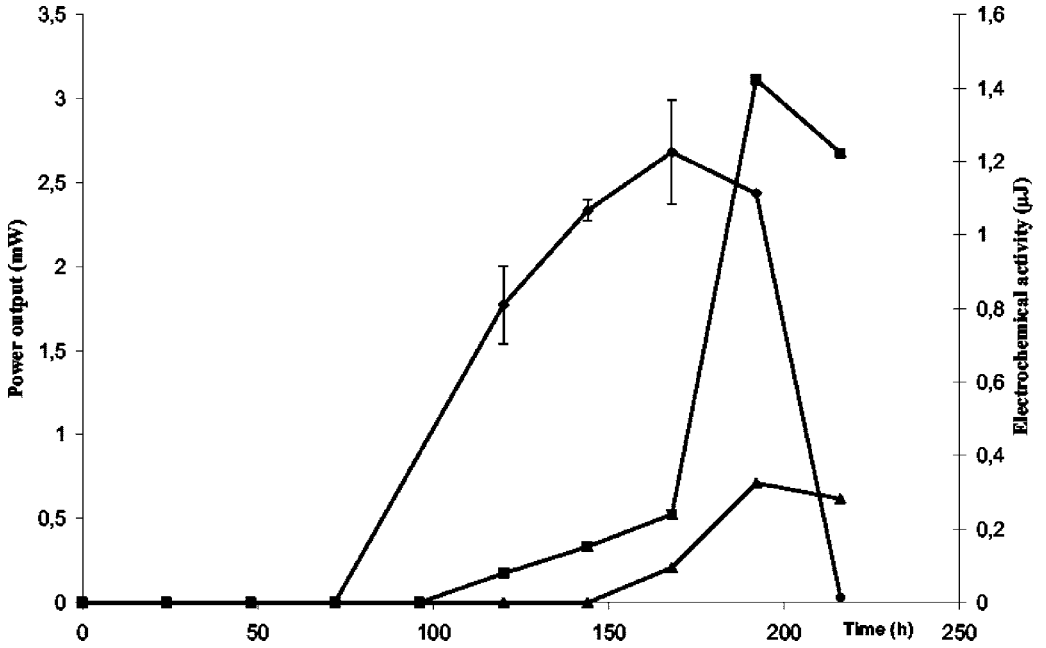

FIG. 5. Evolution in a biofuel cell of the electrochemical activity of the bacteria (■) in relation to the power output (♦). The electrochemical activity of the biofuel cell increased as the power output of the biofuel cell increased. The anaerobic batch reactor exhibited this electrochemical activity only to a very limited extent (▲). In some cases, the error bars were smaller than the symbol.

growth. These pure cultures yielded lower power outputs than the mixed consortium; on average the values were 23.3 ± 6.2 W m^{-2} of anode for *P. aeruginosa*, 4.9 ± 1.8 W m^{-2} of anode for isolate KRA1, and 28.4 ± 2.3 W m^{-2} of anode for isolate KRA3. The bacterial metabolism during the test run was notably affected by the presence of an electron-accepting anode (Table 2).

The bacteria grown in microbial fuel cells had an electrochemical activity which was up to five times greater than the electrochemical activity of bacteria grown in an anaerobic reactor without an electrode. When cyclic voltammetry was performed daily for *P. aeruginosa* grown in the biofuel cells, increasing differences between the peaks on the voltammograms of the centrifuged and noncentrifuged cultures were noted through time. This trend was not observed for the culture grown anaerobically in the serum flasks. The *P. aeruginosa* culture did not grow in the anaerobic serum flask, as expected, and showed little electrochemical activity compared to the biofuel cell analogue. While the electrochemical activity of the peak on the voltammograms of the biofuel cell-grown cultures reached 2.19 ± 1.01 μJ, the electrochemical activity of the anaerobically grown culture reached 0.46 ± 0.01 μJ. The differences between centrifuged and noncentrifuged cultures were minor for this anaerobic control.

DISCUSSION

During the enrichment period, the power output of the biofuel cell increased from 0.6 W m^{-2} of anode surface to a maximum of 4.31 W m^{-2} of anode surface. The microbial consortium shifted toward bacterial strains, which accumulated fewer organic acids and were more electrochemically active.

Microbial fuel cells enhance the growth of bacteria that can use the electrode as an electron acceptor. In a microbial fuel cell, bacteria have limited options for their final electron acceptor. They can either use the electrode or produce reduced metabolites, such as methane or hydrogen gas. The potential of the anode is the potential of the cathode minus all the potential losses and overpotentials in between. This means that the anode potential is generally greater than 0 mV compared to the potential for a standard hydrogen electrode, as previously reported (4). Thus, due to the larger difference in redox potential, bacteria using the anode as an acceptor gain more energy when they use the electrode as an electron acceptor than when they use protons or CO_2. In this way, the system actually selects for bacteria that use the electrode.

Cyclic voltammetry demonstrated how the electrochemical activity of the bacteria is governed by the excreted metabolites. For *P. aeruginosa* and isolate KRA3, the voltammograms obtained without excreted metabolites demonstrated that there was limited electrochemical activity compared to the activity with metabolites. For the other two bacteria tested the change in medium had a minor effect, while the organisms were still electrochemically active, as shown by the roughened shape of the voltammograms. Furthermore, electrochemical activity related to the bacterial membrane was observed by voltammetry of suspended bacteria without metabolites. These data, together with the data from voltammetry of the mixed consortium, indicated that there were two mechanisms for extracellular electron transfer in the mixed consortium: (i) production of extracellular electron shuttles and (ii) components associated with the bacterial cell wall. Indirect evidence for the first mechanism has been provided by Childers et al. (8), who described the ability of *Geobacter metallireducens* to produce electron shuttles that deposit electrons onto Fe^{3+}. This mechanism, however, was not always present in the biofuel cells,

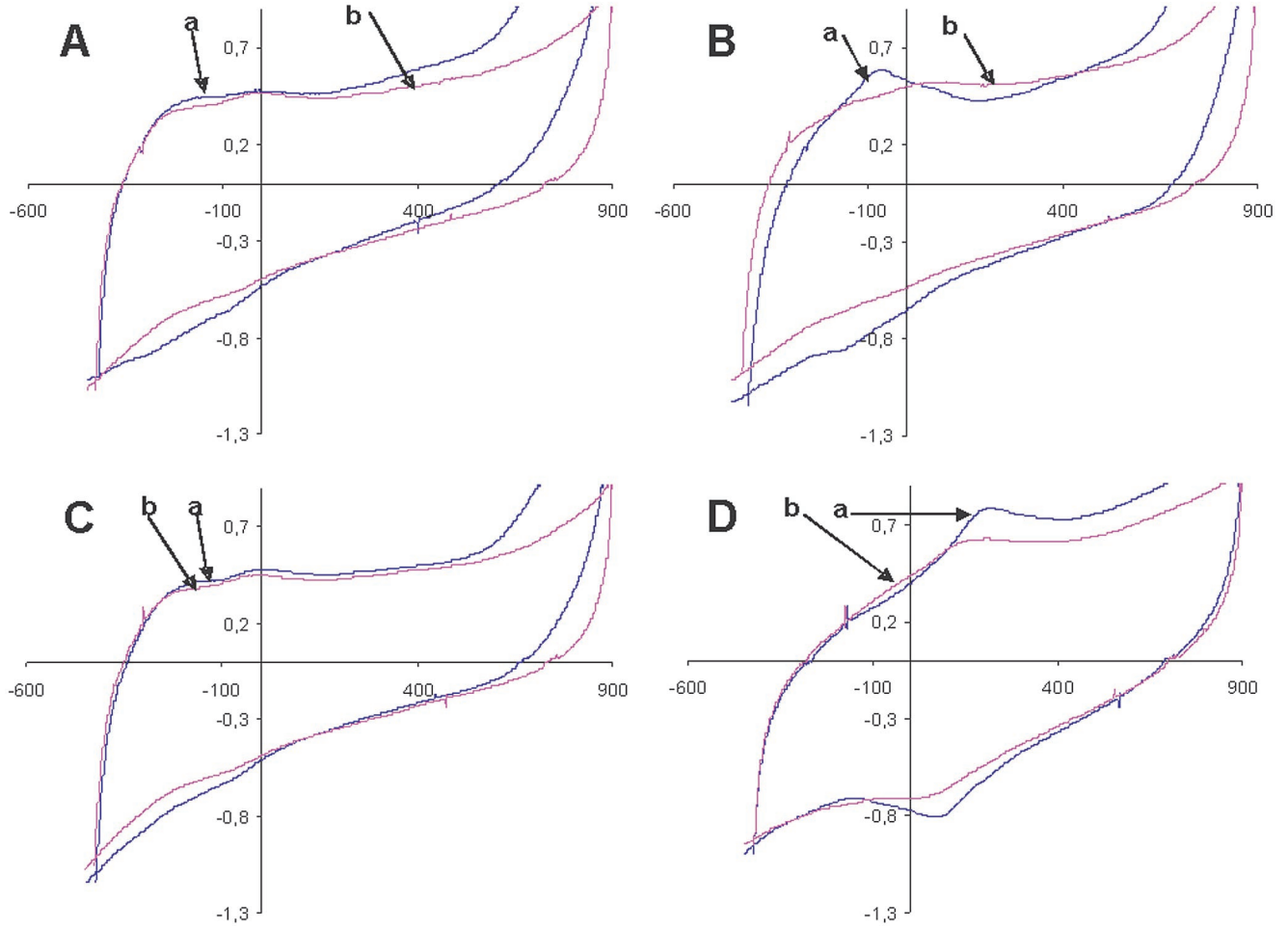

FIG. 6. Cyclic voltammograms of the pure, stationary-phase cultures in spent broth (lines a) and after centrifugation and resuspension in physiological solution (lines b). (A) *Pseudomonas* sp.; (B) *P. aeruginosa*; (C) isolate KRA1; (D) isolate KRA3. *P. aeruginosa* and isolate KRA3 differed from the other bacteria by the disappearance of the dominant peak when the culture was centrifuged and resuspended.

which greatly affected the power output but not the electron transfer efficiency. The second mechanism is supported by the recent findings of Bond and Lovley (4) and Chaudhuri and Lovley (7).

Some of the isolated bacteria are capable of producing compounds that can be regarded as redox mediators. The best-studied organism with respect to redox activity is *P. aeruginosa*, which is capable of producing pyocyanin and several more shuttling compounds (28). This species was previously described for use in a biofuel cell (17, 20). The fact that this facultative anaerobic species is capable of growing in a biofuel cell demonstrates the feasibility of using the electrode as an electron acceptor. Redox interactions can also occur between different bacteria. Electron exchanges have been described

TABLE 2. Reactor concentrations after incubation of axenic bacterial strains for 7 days[a]

Organism	Incubation mode	Final CO_2, a concn (%)	Final concn of volatile fatty acids (mg liter^{-1})	Final no. of CFU ml^{-1}
Pseudomonas aeruginosa	Biofuel cell	14.7 ± 7.6	1,496 ± 371	$3.11 \times 10^6 \pm 0.19 \times 10^6$
	Serum flask	3.5 ± 3.7	145[b]	3.35×10^{5b}
Alcaligenes faecalis	Biofuel cell	13.3 ± 4.7	1,480 ± 397	$2.79 \times 10^6 \pm 0.20 \times 10^6$
	Serum flask	8.3 ± 3.1	84[b]	7.63×10^{5b}
Enterococcus gallinarum	Biofuel cell	18.6 ± 5.1	856 ± 297	$4.03 \times 10^7 \pm 1.09 \times 10^7$
	Serum flask	2.6 ± 0.7	25[b]	9.04×10^{6b}

[a] Gas concentrations are expressed as percentages of the headspace.
[b] Data from a single reactor.

between excreted plastocyanin, produced by *Alcaligenes faecalis* (37), and an azurin produced by *P. aeruginosa* (25). Plastocyanin is a copper-containing blue protein that functions as a mobile electron carrier between cytochrome and photosystem 1 in oxygenic organisms. These findings, together with the voltammetry performed with the isolates, indicate that there is electrochemical activity in suspension, or at least redox controlling properties, in both species. Furthermore, DGGE band sequencing yielded sequences similar to the sequences of several previously described electrochemically active bacteria. *Lactobacillus rhamnosus*, producing microcin (40, 41), and the nicin-producing organism *Lactobacillus lactis* subsp. *lactis* (40) were described previously. *Enterococcus gallinarum*, producing enterocin 012 (19), is suspected to be electrochemically active (G. T. L. Kim, personal communication). Several *Clostridium* species have been reported to have redox activity (24, 33).

The results of the gas measurements and the concomitant bacterial evolution indicate that hydrogen production and electricity generation are mutually exclusive in biofuel cells. Some bacteria unable to transfer electrons to the electrode reduce protons to drain off their electrons in a microbial fuel cell. Direct reduction of protons at the electrode surface is thermodynamically highly unlikely at the low potential present and is moreover hindered by the high overpotential toward a noncatalyzed electrode that exists (36). When isolated cultures were grown without an electrode, the hydrogen production capability was confirmed for most species. This correlates with the identification of the bacteria observed in the bacterial association. The fact that clostridia can produce major amounts of hydrogen is well documented (39). The fact that other identified strains can generate trace amounts of hydrogen has not been reported previously.

The potential losses in an active biofuel cell, which decrease the output, are dependent on several factors: (i) the overpotentials at the anode, (ii) the overpotentials at the cathode, and (iii) the internal resistance of the fuel cell system. The overpotentials can be calculated by using the derived equation of Butler-Volmer (2) and decrease with increasing electrode surface and addition of mediator components.

So far, electron transfer without added mediators has always been described as not feasible due to the high overpotentials at the electrodes (18). Indeed, when bacteria were added as a suspension to a biofuel cell free of a mediator, the results in the short term were limited (32). When electron mediators were added, the power output increased significantly (10, 27, 31) due to the decrease in overpotential at the anode. However, some of the bacteria identified in the consortium selected in the long term have been described as electrochemically active or are known to produce electrochemically active redox compounds. The optimization observed during long-term experiments, therefore, depended on (i) the increase in self-mediating capacity of the bacteria and (ii) the attainment of a critical bacterial concentration above which electron transfer to the electrode was facilitated. This facilitation was due to an increase in electrolyte conductivity and bacterial catalytic activity. The quantities of a microbially produced mediator were low in the short term, but if a reactor was operated for a longer time (as in this study), significant increases in power output were repeatedly noted, together with increases in mediator production (Fig. 3). The formation of these compounds was stimulated by the conditions of the microbial fuel cells (Fig. 4). The possibly higher mediator concentrations could have been caused by the constant oxidizing, neutralizing effect of the electrode. This forced the bacteria to overproduce their often cycled (17) redox components, while the bacterial concentrations increased. These differences appeared to correlate with an increase in the power output of the microbial fuel cell. However, research concerning the relationship among bacterial density, mediator concentration, and power output in microbial fuel cells with mixed bacterial consortia is needed.

While the power output was high for a considerable amount of time, the microbial community analysis demonstrated that the bacterial populations changed with time. This does not necessarily imply that the consortium cannot function in a stable manner (12). However, for future research, chemostatic operation appears to be advisable in order to minimize the variance in reactor parameters.

Reactors in which no formation of soluble mediators was observed had a lower power output but the same efficiency. Furthermore, the findings for the two separate types of biofuel cells (biofuel cells with attached bacteria and biofuel cells with suspended bacteria) demonstrated the ability of both suspended and attached bacteria to perform efficient electron transfer toward the electrode. When the cultures were grown in an anaerobic batch reactor, the electrochemical activity of the bacteria, as measured by voltammetry, was considerably lower (Fig. 4). The pure cultures tested suggested that there was production of the soluble redox mediators, which increased the electrochemical activity of bacteria isolated from biofuel cells, as verified by cyclic voltammetry (Fig. 6).

In conclusion, this study showed that microbial communities in fuel cells evolve specifically, resulting in an optimized bioelectrocatalyzer. Both membrane-bound electron transfer and transfer through soluble redox mediators were observed for the bacterial species analyzed. The bacteria facilitated the extracellular electron transfer and reduced the overpotential at the anode, thereby increasing the power output. The fact that bacteria can produce, and moreover use, redox mediators as electron shuttles enables them to thrive in a microbial fuel cell. While the redox conditions in a microbial fuel cell are disadvantageous for an aerobic bacterial species, such as *P. aeruginosa*, this species can become dominant. Research is needed to further elucidate the microecological significance of these findings. If the mediators are produced in considerable quantities, other bacteria could use them as electron shuttles (16). Moreover, not only could bacteria growing on a surface use the surface as an electron acceptor, but also the more distant layers of the biofilm would be in contact with the anode.

From a reactor-technical point of view, the bacteria constitute a consortium capable of generating a stable power output. Cyclic voltammetry showed its added value for research into bacterial redox behavior and for characterization of redox active components during the study performed. Further refinement and in-depth study of this technique should enable implementation of this technology in a wide variety of (micro)biology-related research fields.

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